Effect of Insulin on the Regulation of Na/K/Cl Cotransporter (NKCC2) in HEK293 Cells

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Jin-Ill Lee

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Abstract

Effect of Insulin on the Regulation of NKCC Cotransporter (NKCC2) in HEK293 Cells

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(Directed by Prof. Syng-Ill Lee, D.D.S., Ph.D.)

Insulin is one of the regulators involved in Na^+ reabsorption in kidney. The Na^+ channel and Na/K/2Cl cotransporter (NKCC2) system are primarily considered as the possible routes for Na^+ movement into the cell. The purpose of this study is to understand the regulation of NKCC2 as a candidate for Na^+ movement across the kidney cell in response to insulin. In order to clarify the role of the insulin in human embryonic kidney cell line, HEK293, the cells were exposed to the different circumstances (isotonic, hypertonic), and then cell volume, expression of NKCC2 mRNA, intracellular Ca^{2+} and functional activity of the cotransporter using pH dye, and the intracellular Ca^{2+} level were analyzed in each condition. The cell volume of HEK293 which was shrunken by hypertonic stress recovered to

nearly original state by the addition of 1 μM insulin. It is obvious that insulin prevented cell shrinkage by operating the regulatory volume increase (RVI) machinery of the cell. However, insulin-induced RVI was blocked by the treatment of 10 μM bumetanide. Furthermore, NKCC2 mRNA was expressed remarkably in the presence of 1 μM insulin. Such results might suggest that the volume regulation of HEK293 cell was, at least, in part mediated through bumetanide sensitive NKCC. On the other hand, the HEK293 cell showed the typical change in intracellular Ca^{2+} concentration in response to carbachol as seen in other normal cells. However, insulin did not induce the change of intracellular calcium level of the HEK293 cell. Taken all together, these findings suggest that the insulin stimulates the sodium transport of HEK293 cell through the upregulation of NKCC2 under the hypertonic condition.

Key words : insulin, NKCC2, HEK293 cell, cell volume, hypertonicity

I. Introduction

The first step of urine processing starts in Bowmanns capsule, the functional unit of nephron. Virtually all plasma except proteins passively filter into glomerular tubule. Then most of the ion is reabsorbed into blood. Defonso *et.al.* (1976) reported that insulin promoted Na^+ reabsorption in the above mentioned process. Naturally, question arises about the route of this insulin mediated increase in Na^+ reapsortion. The possible candidate machinery for the effects are Na^+-glucose cotransporter and Na/K/2Cl cotransporter (NKCC). Type one of NKCC is found in almost all kinds of cell but type II of NKCC is found exclusively in Kidney. Of the two candidates mentioned above, Na-glucose cotransporter reabsorbs sodium with glucose from basolateral side into blood side in proximal tubule. NKCC reabsorbs sodium with chloride and potassium in an electrically silent way.

Among the cell membrane proteins, NKCC is found in both secretory and non-secretory epithelia. Although NKCC is known to mediate the transmembrane movements of Na^+, K^+, and Cl^-, its function is different depending on types of cell (O' Grady, 1987; Haas, 1989; Geck *et. al.*, 1980; Breitwieser *et al.*, 1990; Turner *et. al.*, 1993). For example, the cotransporter contributes to

regulation of salt and water in excitatory tissues, red blood cell and most of the cultured cells. On the other hand, NKCC2 is used mainly to import Cl^- in Cl^- secretory or Cl^- absorptive epithelia. In addition to NKCC2, Cl^- channel, K^+ channel and Na^+/K^+ pump is known to be closely inter-related in intracellular Cl^- movement (Silva *et. al.*, 1977). Cl^- movement via NKCC2 has been reported in kidney and rectum. The NKCC2 is essential in NaCl secretion in avian salt gland (O'Grady *et. al.*, 1987). Likewise, the NKCC is crucial in salivary secretion in that it regulates salt movements and obliged fluid absorption and secretion. Besides, it is important in salt absorption in kidney and maintenance of intracellular Cl^- ion concentration in nerve cell. As mentioned above, understanding about the cotransporter and its regulation mechanism is essential for studying signal transduction system (Turner *et. al.*, 1986; Haas, 1994; Payne and Horbush, 1995).

As it has been mentioned, each transport cycle of NKCC is electrically silent. Which means that the sum of the cation crossing the membrane is same as the sum of the chloride ions crossing the membrane. The transport stoichiometry is 1Na/1K/2Cl in most cells. As all three ions are used, the lack of any of the three cotransported ions leads to down regulation of the cotransporter (O'Grady *et. al.*, 1987; Haas, 1989; Geck and Heinz,

1986).

Several studies have shown that the location of cotransporter and its sensitivity to furosemide (a kind of loop diuretics), differs among tissue types. The secretory NKCC1 cotransporter is located at basolateral side, whereas the absorptive one is located on apical side of the cell membrane. The cloning of cDNA of 5260 bp by Xu *et al.* (1994) gave a breakthrough in the study of the cotransporter (Xu *et. al.*, 1994). Based on such studies, Xu *et al.* suggested that there are 1191 amino acids with 12 transmembrane domains having both N- and C-terminal inside the cell. When this cDNA was transfected into human embroyonic kidney cell line (HEK293), there was ten fold increase in $[^{86}Rb^{+}]_i$ which is dependent on Na^{+} and Cl^{-}. Northern blot analysis showed 7.4 kb mRNA, which was found also in brain, colon, heart, liver and rectal gland (NKCC1). A 5.2kb mRNA was found exclusively in kidney and named NKCC2 by Payne *et al.* (1994).

The activation mechanism of the cotransporter is not revealed clearly as yet. It can be said that at least the actin cytoskeleton is involved in activation of NKCC (Matthews *et. al.*, 1992; Matthews *et. al.*, 1994; Payne *et. al.*, 1995; Wu *et. al.*, 1994). In addition to volume change, other factors have been known to affect the control of the cotransporter. Then what kind of factor

activates NKCC and how it is regulated? It has been found that cAMP or hormones that activate protein kinase C (PKC) and protein phosphatases (Lytle and Forbush, 1992; Pewitt, 1990a, b; Leung *et. al.*, 1994; Paulais and Turner, 1992; Palfrey and Pewitt, 1993; Klein and O'Neill, 1993) influence activation of the cotransporter. There are several reports showing that stimuli that provoked the increase in the cotransporter activity also increased phosphorylation of the cotransporter. In the study using nasal mucosal culture cell from the patient of cystic fibrosis (the cells of a patient with cystic fibrosis is defective of Cl^- channel which is activated by cAMP), it was found that though there was not cyclic AMP mediated Cl^- channel, purinergic receptor mediated Cl^- channel and NKCC was found.

Recently, the phosphorylation site by PKA has been found in human colon NKCC and the regulation mechanism by direct phosphorylation/activation has been suggested at least in human (Payne *et. al.*, 1995; Payne and Forbush, 1994). The activation of the cotransporter by phosphrylation- dephosphorylation has been proposed at first by Altamirano *et al.* (1988). According to their report, when giant squid axon pre-treated with vanadate or fluoride (both are phosphatase inhibitor) was suspended in ATP free medium, the activation of NKCC was extended. As more direct evidence, the phosphorylation itself regulated the

cotransporter activity (Altamirano, 1988).

Since it has been found that if the Cl^- channel at the apical membrane of the rectal gland tubule is blocked, the phosphorylation of the NKCC also was decreased, the secondary modulation by the cotransporter has been suggested (Lytle and Forbush, 1992). It means that it is not certain if the activation of cAMP of the NKCC is the result of direct activation of cAMP-dependent protein kinase (PKA) or that of other secondary kinase. Lytle and Forbush (1992) suggested that phosphorylation of rectal gland cotransporter by secretagogues is rather an indirect result of change in intracellular chloride concentration, than a direct effect of PKA. These Cl-coupling hypothesis is supported by the fact that there is not a PKA phosphorylation site in sNKCC1 (Xu *et. al.*, 1994). In addition, the experiment with rectal gland tubule incubated in elevated K^+ concentration showed that when Cl^- relaease through apical membrane were blocked, there was not phosphorylation of the cotransporter by forskolin. Thus, phosphorylation of NKCC is closly interrelated with Cl^-. However there are differences in interpreting the activation mechanism of the cotransporter. One group suggests that once Cl^- channel is activated, cell shrinkage is followed by the low intracellular Cl^- ion concentration. Thus in order to restore cell volume, the NKCC is directly phosphorylated. Others suggest that

decreased Cl^- concentration itself directly activates the cotransporter. On this aspect, primary concern has been given to the transport process of the extracellular Cl^- and its modulation, in Cl^- secreting gland cells.

Diabetes, which is commonly involved with insulin, can be divided into two types. Type one is insulin independent and occurs about 5 to 10 percent of patients. Remaining 95 to 90 percent of diabetes patients fall into type II, recently known to be involved with resistin (Steppan, 2001). In dental diseases, diabetes complicate wound healing and control of periodontal disease. Insulin has various effects in human body. Besides the effect on ion transport, which is the main concern of this study, it has metabolic effects including metabolism of carbohydrates, protein and lipids and growth promoting effects on DNA synthesis and cell division. It's action is initiated in membrane receptor, followed by increase in tyrosine kinase activity of β -subunit and autophosphorylation, resulting in sequential phosphorylation or dephosphorylation of other inctracellular proteins. As it is well known that increase in intracellular Ca^{2+} is crucial in activation of the NKCC2, there is the possibility that insulin can influence in activation of the cotransporter in HEK293 cell. In regards to diseases, the role of the cotransporter in relation to the cell volume has been limited in hypertension.

However, as the clinical report says, from 50% to 70% of diabetes patients finally suffers from kidney disease. Thus the role of the cotransporter in kidney should not be overlooked and we hope that if the present study should help in revealing the role of the cotransporter, it will provide important data for the relationship between diabetes and NKCC2.

In the present study, we observed how cotransporter is regulated by insulin in HEK293 cell. It is based on the hypothesis that insulin effect on Na^+ reapsortion is mainly due to NKCC2 activity. Thus we focused on NKCC2 and recorded the change of its activity. The cells were exposed to normal and hypertonic condition, observed expression of NKCC2 mRNA, intracellular Ca^{2+} and functional activity of the cotransporter using pH dye in each condition to find out 1) the effect of cell volume in activation of the cotransporter, 2) expression of NKCC2 mRNA, and 3) how insulin makes effects on expression of NKCC2 and its fuctional activity on cell volume.

Ⅱ. Materials and methods

1. Materials

All routine cell culture media were obtained from GIBCO/BRL (Grand Island, NY). Insulin was purchased from Sigma Chemical Co., Ltd. (St. Louis, MO). ICR mice were purchased from Samtacho Co., Ltd. (Seoul, Korea). All other chemicals were of the highest grade commercially available.

2. Methods

Of the two regulation mechanism of NKCC2 (metabolic and non-metabolic), we expected cell volume change by metabolic regulation. These volume change is one of the controlling factor regulating cell function. It also is involved in protein synthesis and degradation and is known to affect gene, enzyme, carrier and messenger. We therefore expected change in mRNA expression of HEK293 cell after stimulating with insulin. In the present study we examined how insulin works on the transporting system which is widely used for control of diabetes.

(1) HEK293 cell cuture

Human Embryonic Kidney cells (HEK293) (ATCC, Manassas, VA) which were stored at -86 ℃ were released in 37

℃ water bath, incubated in 10 ml culture media (DMEM, penicillin-streptomycin, Newborn calf serum, L-glutamine), and then dispersed by forcing through polypropylene pipettes with decreasing orifice. Then the cells were centrifuged at 1000 rpm for 10 minutes. After removing the supernatants, the cells were prepared for culture dish and cultured in 37 ℃, 100% humidity, 5% CO_2. Culture media was changed every 3 day, and carefully managed to grow cell in monolayer

(2) Measurement of cell volume

The coverslip on which cell were cultured was mounted on a chamber, which was set on the mechanical stage of an inverted microscope connected to a video-imaging system. The cells were perfused with insulin-containing solution for 10 min before the experiments. Images of the cell through optical microscope were continuously recorded with a video image system. To estimate cell volume, the area of the HEK293 cell in the video image was measured.

(3) Measurement of intracellular pH and Ca^{2+} concentration

In order to stimulate the cell and obtain the cellular responses, perfusion system was used. HEK293 cells were loaded with fura-2 by incubation with 2 μM fura-2/AM in

HEPES-buffered solutin (mM; NaCl 110, KCl 4.5, NaH_2PO_4 1.0, $MgSO_4$ 1.0, $CaCl_2$ 1.5, $NaHCO_3$ 25, HEPES-Na 5, HEPES free acid 5, D-glucose 10; equilibrated with 95% O_2, 5% CO_2 to give a pH of 7.4). The cells were allowed to attach to a coverslip which formed the base of a cell chamber mounted on the stage of inverted microscope (Nikon, Japan), and $[Ca^{2+}]_i$ was measured in single cells by spectrofluorometer (Photon Technology International, Brunswick, NJ, USA) with excitation at 340 nm and emission measured at 510 nm (Fig. 1). The measured values represent as a ratio of 340/510.

The cells were perfused at the flow rate of 2 ml/min with perfusion pump (Micro Tube Pump, MP-3) during the pH and intracellular $[Ca^{2+}]_i$ were measured. The pH and intracellular $[Ca^{2+}]_i$ were recorded continously using the personal computer. To keep the temperature 37 ℃, perfusion system was equipped with water jacket.

(4) Preparation of perfusion solution

The functional activity of NKCC2 was measured. The composition of perfusion solution was shown in Table 1. NH_4^+ pulse (20 mM) was challenged to give a alkaline shock to the cell. In the case of alkaline shock (20 mM NH_4Cl), 20 mM NaCl was removed instead of adding 20 mM NH_4Cl, to keep the

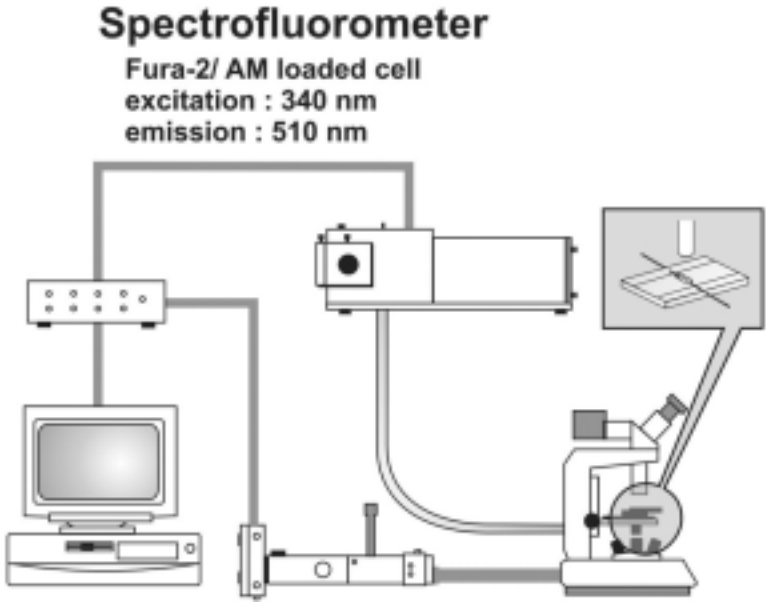

Fig. 1. Schematic diagram of Spectrofluorometer for the mesurement of intracellular pH and Ca^{2+}.

Table 1. Composition of perfusion solution (mM)

	pH	Ca^{2+}	High K^{+} solution
NaCl	127.5	110.0	0.0
KCl	4.5	4.5	132.0
NaH_2PO_4	1.0	1.0	1.0
$MgSO_4$	1.0	1.0	1.0
$CaCl_2$	1.5	1.5	0.0
HEPES-Na	10.0	5.0	10.0
HEPES	10.0	5.0	10.0
$NaHCO_3$	0.0	25.0	0.0
D-Glucose	10.0	10.0	10.0

osmolarity to the physiological condition. While test was performed in Ca^{2+} free solution, making nominally zero Ca^{2+} by adding 1 mM EGTA. Perfusion solution was kept at 37 ℃ in water bath.

(5) hNKCC2 mRNA expression

RNA extraction, quantitation, and RT-PCR was executed by the method described in Sambrook *et al.* (1989) with slight modification.

① Extraction of total RNA

Insulin was treated into the culture dish. in which cell grows in the form of monolayer. Response to the insulin was checked as a function of time and doses. For isolation of RNA from HEK293 stimulated by insulin, cells were lysed using Trizol. That is, after the completion of the reaction by insulin for the given period of time, medium was discarded followed by two times washing for elimination of remaining PBS. One ml of Trizol was mixed with the cells, and passing the cell lysates several times through the pipette. Another 1 ml of Trizol added to the pretreated cells to make them completely solubilized. A Trizol treated cells were kept in room temperature for 5 min, and mix it thoroughly with same volume of chloroform, then stand it on

the bench for another 3 min. Spin it at 12,000×g for 15 min, take the supernatant and move the sample to the new tubes and add the same volume of isopropanol, allowing 10 min reaction on the bench for 10 min. It was spinned at 12,000×g and the pellet was suspended in 1 ml of 75 % ethanol. The final suspended pellets were stored in at -70 ℃ until next experiment. All plastic and glassware was autoclaved, all buffers were made RNAase free by treatment with 0.05 % DEPC or purchased RNAase free.

② RNA quantitation and visualization

Aliquot in 75% ethanol was centrifuged at 15,000 rpm for 5 min. And extracted RNA was dissolved in 50 ㎕ DPEC water. RNA concentration was measured by UV Spectrophotometry at 260 nm. RNA quantity was quantitated in ìg/ml.

③ cDNA synthesis and RT-PCR

One ㎍ of total RNA was taken and samples for PCR were mixed with 1 ㎕ of oligo (dT) 18 primer and made up to 12.5 ㎕ with sterile water treated with DEPC. Mixtures were heated at 70¡℃ for 5 min. and cooled down. After snap centrifugation Reaction buffer, dNTP mix, recombinant RNase inhibitor, MLV reverse transcriptase were added. Mixture were incubated at 42 ℃ for 1 h and 94 ℃ for 5 min to stop the synthesis of cDNA.

cDNA synthesized placed on ice for immediate use, or stored at -20¡ ℃.

④ Polymerase chain reaction (RT-PCR) and electrophoresis

Oligonucleotide primers were synthesized by gene tech PCR amplification was performed in a thermal cycler on 2 ㎕ of cDNA in a reaction volume of 30 ㎕ containing 2 mM $MgCl_2$, 200 μM each deoxynucleoside triphosphate, 0.2 μM each primer. Amplification was carried out using 29 cycles of 94 ℃ for 5 min. When the reaction mixture was reached up to 80 ℃, ampli-Tag enzyme was added, followed by another reaction at 94 ℃ for 30 sec, 63 ℃ for 30 sec, and 72 ℃ 1 min. Again, DNA extension was done reacting at 72 ℃ for 7 min. The synthetic cDNA, buffer, dNTP, $MgCl_2$, primers (upper primer : 5'-GCA TTT GAT TTT GAG ATT GGC GTG GTT-3', lower primer ; 5'-TTT GGC CTA ATG TTG ATG TCA CCG ATG ATA-3') put in the PCR tube, and then was reacted at 94 ℃ for 5 min.

Following amplification, PCR products were visualized by electrophoresis on 1.5% agarose gels with TBE buffer at 120 volts. A 100 bp ladder was also loaded to allow size estimation.

Ⅲ. Results

1. Volume response of HEK293 to insulin or hypertonic condition

For the preliminary examination, HEK293 cell was treated with insulin and cellular volume change was observed. When the medium containing 1 μM of insulin perfused to HEK293cell, no significant volume change was detected. Cellular volume response of HEK293 cell to hypertonic condition was also observed by perfusing the medium containing 100 mM of sucrose. As shown in Fig. 2 more than 30 % of the cell volume was decreased when HEK293 cell was perfused with 100 mM of sucrose solution in perfusion chamber. When the sucrose solution was substituted with normal medium, HEK293 cell restored its volume after short period of cell shrinkage.

2. Effect of insulin on the regulation of cell volume in HEK293 under hypertonic condition

The effect of insulin on volume regulation of HEK293 cell under hypertonic condition was tested (Fig. 3). Since no significant effect of insulin on cell volume was detected in isotonic condition, the experiment was carried out in hypertonic condition. As shown in Fig. 2, When the hypertonic stress using

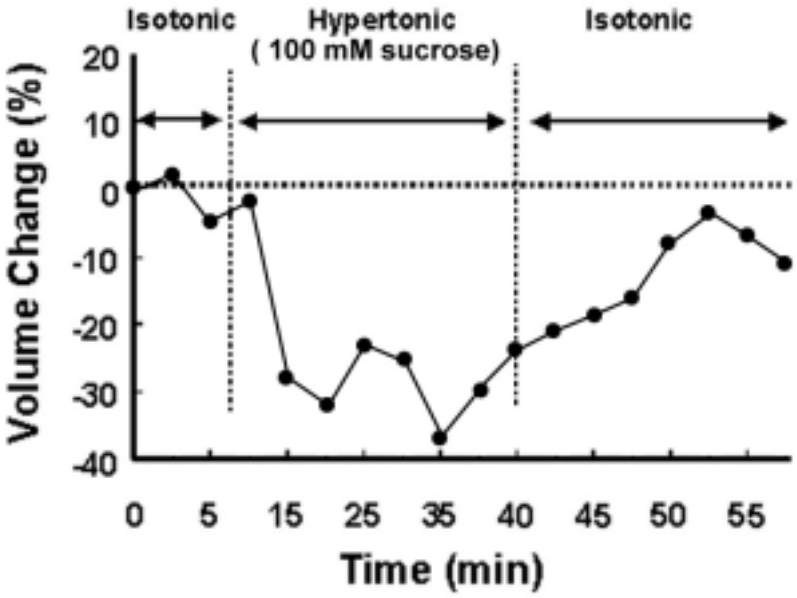

Fig. 2. Volume change of HEK293 cell in hypertonic condition.

The medium containing 100 mM of sucrose was perfused to the chamber which HEK293 cell was mounted. After 30 minutes perfusion, the medium was substituted to normal medium. Cell volume change of HEK293 cell was measured by computerized video-imaging system. The images of the optical microscope were continuously recorded. To estimate cell volume, the area of HEK293 cell in the video image was measured. The averaged value of area measured in the first 2 min was used as the control. The relative cell volume, V/V_0 was estimated, where V is the volume, and subscript $_0$ is the value of the control.

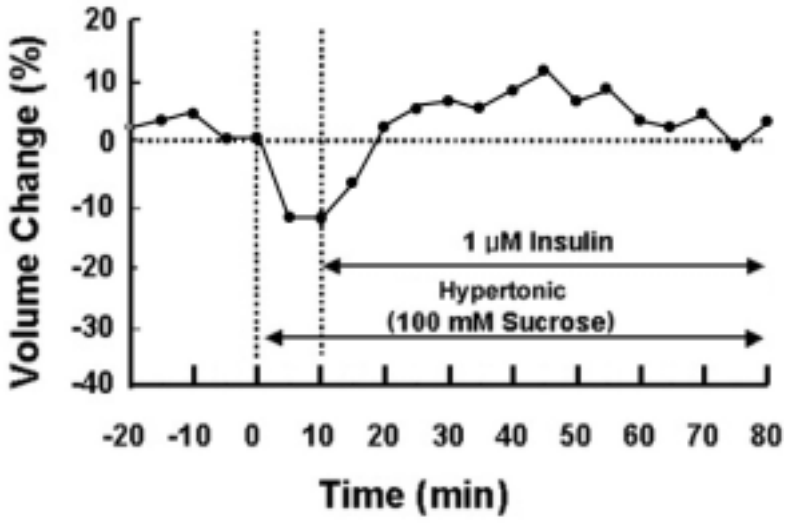

Fig. 3. Effect of insulin on volume change of HEK293 cell in hypertonic condition.

The medium containing 100 mM of sucrose was perfused to the chamber which HEK293 cell was mounted. After 10 minutes perfusion, the medium was substituted to the medium containing sucrose and insulin. Cell volume change of HEK293 cell was measured by computerized video-imaging system. The images of the optical microscope were continuously recorded. To estimate cell volume, the area of HEK293 cell in the video image was measured. The averaged value of area measured in the first 2 min was used as the control. The relative cell volume, V/V_0 was estimated, where V is the volume, and subscript $_0$ is the value of the control.

sucrose containing medium was applied to HEK293 cell, cellular shrinkage was initiated the same manner. After 10 minutes perfusion of hypertonic medium which contains 100 mM sucrose, 1 μM of insulin was added to the hypertonic medium. As insulin containing hypertonic medium was perfused, the volume was recovered to original state. It can be inferred from this result that insulin activated regulatory volume control mechanism of HEK293 cell.

3. Involvement of NKCC2 in regulatory volume increase in HEK293 cell

Since the regulatory volume increase was activated by insulin, it is necessary to identify the main route which insulin may activate the regulatory volume increase. To identify the mechanism bumetanide, which is a specific inhibitor of NKCC, was used. HEK293 cell was applied to hypertonic condition to induce hypertonic shrinkage (Fig. 4). After 20 minutes perfusion of hypertonic medium, 1 μM of insulin and 10 μM of bumetanide were added to the hypertonic medium simultaneously. As shown in Fig. 2, the addition of insulin triggered the recovery of cellular volume in HEK293 cell to the resting stage. However, when insulin and bumetanide was added simultaneously to the hypertonic medium, such recovery was not detected and the

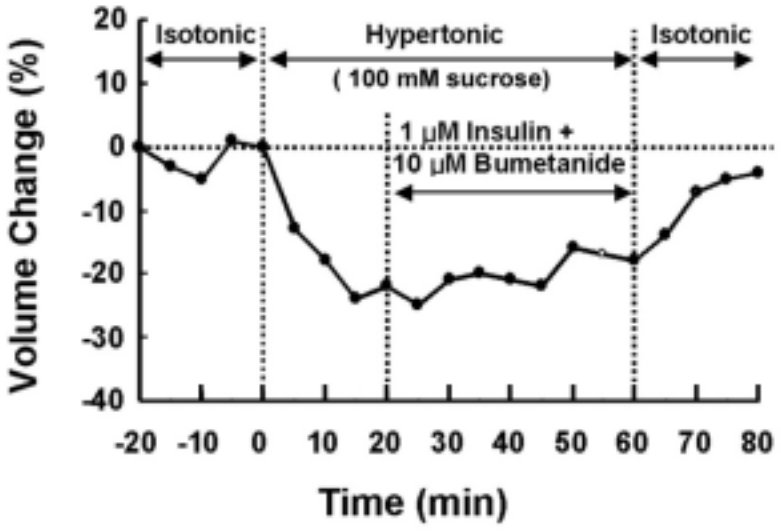

Fig. 4. Volume response of HEK293 cell to insulin and bumetanide in hypertonic condition.

The medium containing 100 mM of sucrose was perfused for 20 minutes to the chamber which HEK293 cell was mounted. The medium was then substituted to the medium containing sucrose, insulin and 10 μM of bumetanide and perfused for 40 minutes. Normal medium was perfused to the chamber for the last 20 minutes to confirm the recovery of cell volume. Cell volume change of HEK293 cell was measured by computerized video-imaging system. The images of the optical microscope were continuously recorded. To estimate cell volume, the area of HEK293 cell in the video image was measured. The averaged value of area measured in the first 2 min was used as the control. The relative cell volume, V/V_0 was estimated, where V is the volume, and subscript $_0$ is the value of the control.

shrinkage of HEK293 cell maintained. The volume of HEK293 cell was recovered to the resting stage when normal medium was perfused the chamber.

4. Activation of NKCC2 by insulin in hypertonic condition

In order to elucidate the effect of insulin on functional activity of NKCC2, pH recovery rate was measured. For this experiment NH_4Cl was added to the medium and perfused to HEK293 cell. As NH_4Cl containing medium perfused to the chamber intracellular pH $[pH]_i$ was rapidly increased. However the intracellular pH was slowly decreased (Table 2). After the medium was substituted to normal medium, the $[pH]_i$ was recovered to the resting stage. This experiment was repeated after 5 minutes perfusion of insulin containing medium. When insulin containing medium was pre-perfused to the HEK293 cell, the decrease of intracellular pH which was increased due to the NH_4Cl perfusion was slightly faster. This result indicates that pre-incubation of HEK293 cell in insulin containing medium might activate NKCC2 activity to recover the intracellular pH.

5. Increase of NKCC2 mRNA expression by insulin

To confirm the fact that insulin has direct effect on NKCC2, mRNA expression of NKCC2 was analyzed with RT-PCR. As the

Table 2. Effects of insulin on the pH recovery in hypertonic conditions.

	Control	Insulin	
		Isotonic	Hypertonic
Rate of pH recovery	- 3.38 ± 1.10	- 2.49 ± 0.35*	- 4.05 ± 1.36

*: Significant difference between control and insulin treated group ($p = 0.00827$). Each figures represent the mean ± standard error. Rate of pH recovery were measured to compare the activity of cotransporter, NKCC2 before and after the treatment of insulin in condition of hypotonic and hypertonic condition. Insulin was added to the HEK 293 cell line for 30 min.

concentration of insulin was increased in isotonic condition the expression of NKCC2 mRNA in HEK293 cell was up regulated till 1 μM of concentration and slightly decreased at 10 μM (Fig. 5). This experiment was repeated in hypertonic condition using 100 mM sucrose. When 1 μM of insulin was added to the hypertonic medium, the expression of NKCC2 mRNA was up regulated as the same manner as in isotonic condition (Fig. 6).

6. Effect of insulin on intracellular Ca^{2+} concentration

Intracellular Ca^{2+} concentration in HEK293 measured using Fura-2. First of all, the response of HEK293 cell to carbachol, one of muscarinic effecters, was recorded. As shown in Fig. 7, typical biphasic pattern of Ca^{2+} change was appeared. The HEK293 cell showed the typical change in intracellular Ca^{2+} concentration in response to carbachol as seen in other normal cells, validating the cell and Ca^{2+} measuring system. The effect of insulin and response to cell volume change was measured and analyzed. As a result, Insulin did not change intracellular calcium level on the HEK293 cell. The change in cell volume (hypotonic or hypertonic condition) did not affect intracellular Ca^{2+} level. These results mean that the mRNA expression change by insulin was not mediated by increase in intracellular Ca^{2+}. On the other hand, there is a report that there was increase in NKCC

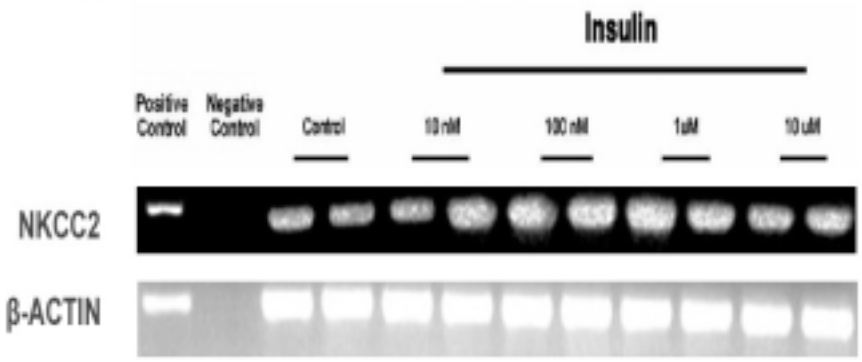

Fig. 5. Effect of insulin on NKCC2 mRNA expression HEK293cell in isotonic condition. Insulin (1 μM) was added to the HEK293 cell culture. After incubation for additional 30 minutes, total RNA was isolated. mRNAs were amplified according to the method described in materials and methods.

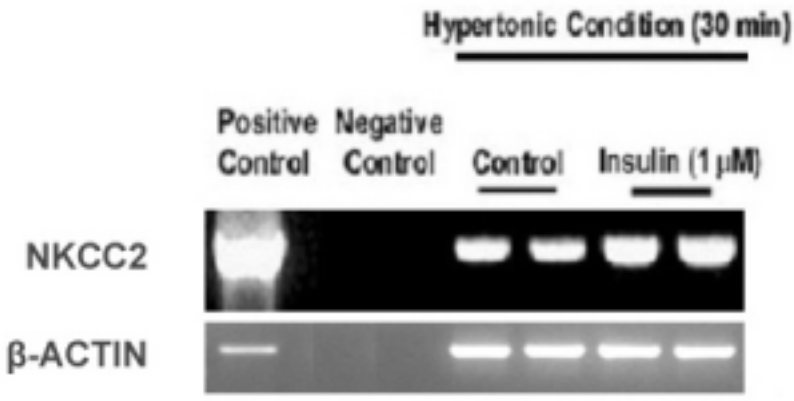

Fig. 6. Effect of insulin on NKCC2 mRNA expression HEK293cell in hypertonic condition. Insulin (1 μM) was added to the HEK293 cell culture in the presence of 100 mM sucrose. After incubation for additional 30 minutes, total RNA was isolated. mRNAs were amplified according to the method described in materials and methods.

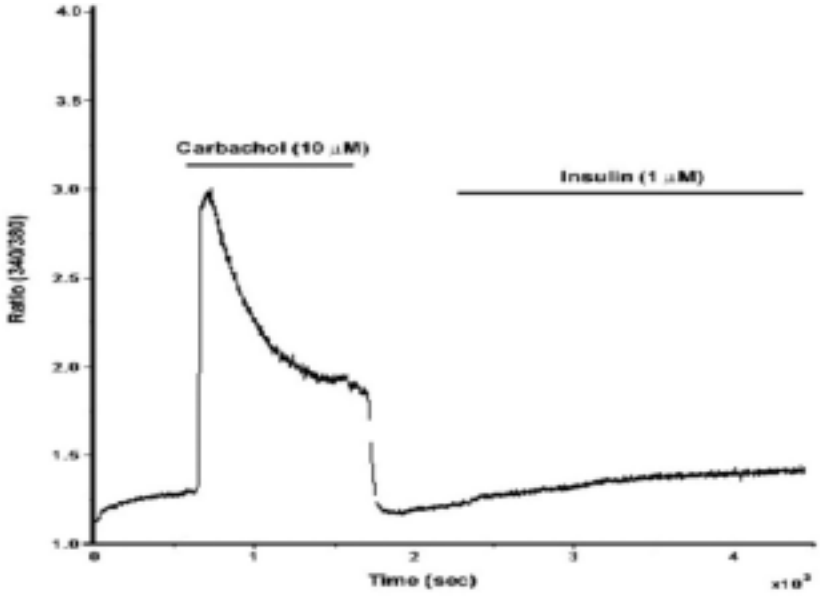

Fig. 7. Effect of insulin on the activity of Ca^{2+} channel of HEK293 cell. Carbachol (10 μM) was perfused to the chamber which HEK293 cell was mounted. The medium was then substituted to the medium containing insulin. Relative fluorescence was recorded using 340 and 380 nm fluorescence filters.

cotransporter activity of NIH3T3 fibroblast in response to stimulation by bradykinin and PKC was involved in the signal transduction process (Sargeant *et. al.*, 1995; Hichami *et. al.*, 1996). Possibly, there might be a difference in signal transduction system between HEK293 cell and fibroblast.

Ⅳ. Discussion

In this study, we have observed the relationship between cotransporter and insulin in HEK293 cell. In physiological condition, renal tubules are exposed to dynamic osmotic environment, ranging from 300 to 1200 mOsmol. Especially, in Henly's loop, which is the part thought to be responsible for NKCC2 activation, osmotic environment is quite hypertonic. One of the main functions of NKCC2 is cell volume homeostasis. The physiological role of NKCC2 in volume control has been studied as part of the function of the cotransporter. In hypertonic condition, as cell volume is decreased, cell tries to recover its original volume by importing ions such as Na^+/Cl^-, so called regulatory volume increase (O' Grady, 1987; Haas, 1989). Na^+/K^+ cotransporter, Na^+/H^+ pump and Cl^-/HCO^{3-} exchanger also contribute to the process. Thus the volume change can be used as a parameter for NKCC2 activity. So we indirectly measured NKCC2 activity by recording volume change. The cells exposed to 100 mM sucrose showed hypertonic shrinkage and the addition of insulin activated regulatory volume increase (RVI) as shown in Fig. 2. There might be several candidates, which turn on the regulatory volume increase of HEK293 cells such as NKCC and Ca^{2+} channels. Among these, we have demonstrated the

involvement of NKCC2 in RVI that is induced by insulin. Fig. 3 showed that bumetanide, which is a specific inhibitor of NKCC, showed inhibitory effect on RVI which was induced by insulin. The result clearly indicates that NKCC2 is the main route of insulin mediated regulatory volume increase in HEK293 cells.

Although there are evidences that NKCC in endothelial cells is activated by intracellular calcium and is inhibited by protein kinases (Klein, and O'Neill, 1993), the mechanism by which cell shrinkage activates cotransporter in a variety of cells is unknown. Breitweiser *et al.* (1990) reported that NKCC activity is dependent on intracellular Cl^- concentration. In his experiment using squid giant axon (Breitwieser *et. al.*, 1990; Hass and Mcbrayer, 1994). They also found that colonic cotransporter is directly activated by PKA (Molony *et. al.*, 1987). At present, not all workers agree that NKCC activity is affected by intracellular chloride concentration. Nevertheless, it must be very interesting hypothesis that NKCC is secondarily activated as intracellular Cl^- concentration is decreased, since it may contribute to understanding about the controlling mechanism about the import and export of Cl^- in salt mobilizing epithelial cell. At present, it can be clearly said that as the cell shrinks as a result of the decreased intracellular Cl^- concentration, this decreased cell volume may act as a secondary activator of the cotransporter

(Payne and Horbush, 1995). Though it has not been clearly found how intracellular Cl^- concentration modulate NKCC, a suggestion that Cl^- binding site might be Cl^- sensitive kinase (or phosphatase) or cotransporter itself.

Aside from the above mentioned studies on secretive type cotransporter, studies about the regulation of cotransporter activity on absorptive type cotransporter (NKCC2) has been done in mouse medullary thick ascending limbs of Henles loop (Sun *et. al.*, 1991; Molony *et. al.*, 1987). On such tissues, NaCl is re-apsorbed as Cl- is transported by cotransporter. And the cotransporter is activated by substances that activates cAMP, like ADH (antidiuretic hormone) (Molony *et. al.*, 1987). Interestingly, a report by Sun *et al.* (1991) showed that chloride importation is done by Na^+/Cl^- cotransporter independent of K^+ under unstimulated condition, whereas Cl^- import was dependent upon K^+ under stimulation with ADH (Sun *et. al.*, 1991; Alvo *et. al.*, 1985; Schlatter and Greger, 1985).

It has been suggested that forskolin reorganizes F-actin, and subsequent structural change in F-actin contributes to the incorporation of cotransporter in vesicular compartment (Molony *et. al.*, 1987 Gamba, *et. al.*, 1994; Shapiro, *et. al.*, 1991). As has been iterated, NKCC is involved in cell volume regulation as well as Cl^- resorption. On some types of cell, cAMP, intracellular

Ca^{2+} concentration or increase of protein kinase C activity contributed to the activation of cotransporter, while on other cell types, non or reverse effect by the same agents was reported (Paulais, and Turner, 1992).

The mechanism of insulin action on cell volume regulation is not known much as yet. Nevertheless, there were reports that insulin activates both Na^{+}-permeable channels (Marunaka, 1996) and the bumetanide-sensitive NKCC (Sargeant *et. al.*, 1995), such activation would likely result in an increase in cell volume. To find out the mechanism of volume regulatory action of insulin under hypertonic condition, we tested the effect of bumetanide on the insulin-induced swelling. In order to elucidate the mechanism how insulin triggers NKCC2, the functional activity of NKCC2 and mRNA expression were measured. After co-application of insulin and bumetanide, the HEK293 cell failed to recover its volume. Bumetanide blocked the insulin effect on volume recovery as seen above in Fig 2 almost completely. So we suggest that at least part of the action of insulin on HEK293 cell volume was mediated through bumetanide sensitive NKCC. When HEK293 cell was pre-perfused by insulin-containing medium, the decrease of intracellular pH that was increased due to the NH_4Cl perfusion was slightly faster than the case of NH_4Cl only. This result indicates that pre-incubation of HEK293 cell in insulin

might activate NKCC2 activity to recover the intracellular pH.

It has been found that NaCl re-absorption increased by the mediation of cyclic AMP. Moreover, decrease in intracellular Cl^-, ADH (increases cAMP), glucagon and isoproterenol are the factor that leads to the activation of the cotransporter and adds to the complexity of its regulation mechanism (Mattews *et. al.*, 1992; Igarashi *et. al.*, 1995; Russel *et. al.*, 1990). The activation mechanism of the cotransporter, from what has been found so far, can be summerised as 1) direct phosphorylation of the cotransporter, 2) secondary regulation effect mediating Cl^- channel, and 3) regulation through cytoskeleton. But, increase in membrane insertion of the cotransporter in cytosol can not be ignored because, 1) when parotid salivary acini of white mouse was stimulated with ALF4 (b-adrenergic agonist), phosphrylation of 175 kD coransporter was closely related to the upregulation 2) whereas, in hypertonic shrinkage or treatment with calyculin A (phosphatase inhibitor) lead to upregulation without phosphorylation of the cotransporter, and 3) whereas carbachol (a kind of muscarinic agonist) induced upregulation of cotransporter accompanied phosphorylation, thapsigargin did not, even though it also mobilizes intracellular Ca^{2+} like carbachol. As it has been reviewed, upregulation of the cotransporter and phosphorylation of it needs not necessarily occur at the same time (Tanimura *et. al.*,

1995). In other words, direct phosphorylation of the NKCC is only a part of the regulation mechanism. Then, 1) the possibility of activation through phosphorylation of membrane related protein other than direct phosphorylation of the transporter, 2) increase in membrane insertion of cotransporter in cytosol should be included in the activation process of the cotransporter.

In concern with the expression of mRNA expression insulin has up-regulated in both isotonic and hypertonic conditions. Under the isotonic condition, insulin increased the NKCC2 mRNA expression though the magnitude was different. On the other hand, the effect of insulin under hypertonic and isotonic condition was different. Insulin induced the expression of NKCC2 mRNA in bothe isotonic and hypertonic condition, however, physiological activity was activated in only hypertonic condition. Therefore, insulin might be considered as an activator of NKCC2 as well as inducer that can increase the expression of NKCC2 mRNA.

In conclusion, though NKCC2 is believed to be involved in cell volume regulation, essentially the cotransporter is involved in both physiologic volume regulatory and volume-sensitive transport mechanism. Insulin clearly increased NKCC2 mRNA expression when cell was shrunken. From the fact that insulin did not mobilize intracellular Ca^{2+}, it was confirmed that Ca^{2+} was not involved in the expression of NKCC2 mRNA gene expression. As

mentioned above, insulin stimulates the sodium transport of HEK293 cells through the upregulation of NKCC2 under the hypertonic condition.

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국문요약

Effect of Insulin on the Regulation of Na/K/Cl Cotransporter (NKCC2) in HEK293 Cells

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이진일

본 연구는 Kidney 세포주인 HEK293 세포에서 인슐린이 sodium 재흡수에 미치는 영향을 살펴보고, 그 기전으로서 NKCC2에 의해 ion transport 가 upregulation 되었을 가능성에 대해 기능적, 유전학적 조사를 수행하였다.

신장에서 인슐린에 의한 Na^+ reabsorption 증가에 대한 보고서가 있는바 그 가능한 기전은 proximal tubule에 존재하는 Na-glucose cotransporter 와 Thick ascending loop of Henry 에 존재하는 NKCC2 를 들 수 있으며 본 연구에서는 insulin에 의한 효과가 주로 NKCC2 에 의한 것이라는 가설을 세우고 여러 조건에서 insulin에 의한 NKCC2 activity 의 변화를 측정하였다.

세포용적 변화를 통한 실험에서 insulin은 고장액 상태에서 세포용적 회복을 촉진시켰으며 bumetanide를 통한 실험으로 이런 효과가 NKCC2 에 의한 것임을 확인하였다. 또한 세포에 insulin을 투여한 후 alkaline shock을 부여하고 spectrophotommeter 를 통하여 pH 회복율을 측정한 결과 고장액에서 recovery rate가 증가하는 경향을 확인하였다.

위에서의 결과를 토대로 유전자 수준에서 이를 확인하기 위해서 각기 다른 농도의 insulin으로 전처리 후 mRNA의 변화를 관찰한 결과 대조군에 비해 뚜렷한 mRNA expression의 증가를 확인하였다.

Insulin을 1 μM 로 고정하고 전처리 시간을 달리하여 mRNA의 발현을 확인한 결과 10 분 경에는 약 5배 이상 증가한 것을 관찰하였다. 이는 insulin response가 비교적 빠른 시간 내에 나타났음을 보여준다. 동일한 실험을 고장액에서 하였을 때, 역시 증가되는 양상을 보였다.

또한, calsium 을 매개로한 신호 전달체계를 확인하기 위하여, Ca^{2+}의 대표적인 agonist인 carbacol로 자극한 후 insulin에 의한 세포 내부 Ca^{2+} 변화를 관찰할 수 없었다. 따라서 인슐린에 의한 반응이 Ca^{2+}을 매개로 한 intracellular signaling pathway를 거치는 것 같지는 않다. 결론적으로 insulin이 renal tubular cell line인 HEK293 세포에서 NKCC2 activity를 증가시키는 것으로 보이며, 특히 고장액에서 NKCC2 mRNA 발현을 증가시켰다.

핵심되는 말 : insulin, NKCC2, HEK293 cell, 세포용적